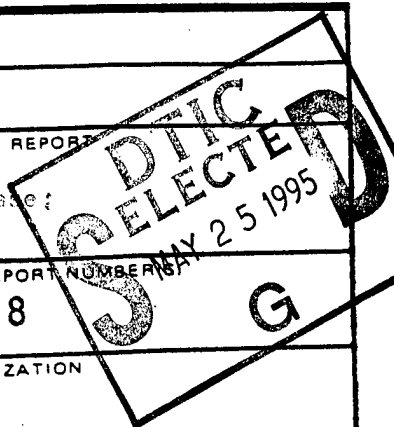


REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED		1b. RESTRICTIVE MARKINGS													
2a. SECURITY CLASSIFICATION AUTHORITY UNCLASSIFIED		3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.													
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER AFOSR-IR-95-0348													
4. PERFORMING ORGANIZATION REPORT NUMBER(S) USAF/DA-3		7a. NAME OF MONITORING ORGANIZATION AFOSR/NL													
6a. NAME OF PERFORMING ORGANIZATION Dept of Physical Sciences Southeastern Okla State Univ	6b. OFFICE SYMBOL (If applicable) SEOSU/CHEM	7b. ADDRESS (City, State and ZIP Code) 110 Duncan Ave Suite B115 Bolling AFB DC 20332-0001													
6c. ADDRESS (City, State and ZIP Code) Department of Physical Sciences SEOSU, Mail Station A Durant, OK 74701-0609		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F49620-94-1-0014													
8a. NAME OF FUNDING/SPONSORING ORGANIZATION AFOSR	8b. OFFICE SYMBOL (If applicable) NL	10. SOURCE OF FUNDING NOS. <table border="1"><tr><td>PROGRAM ELEMENT NO.</td><td>PROJECT NO.</td><td>TASK NO.</td><td>WORK UNIT NO.</td></tr><tr><td>61102F</td><td>2312</td><td>AS</td><td></td></tr></table>		PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.	61102F	2312	AS					
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61102F	2312	AS													
8c. ADDRESS (City, State and ZIP Code) AFOSR/NL 110 Duncan Ave., Suite B-115 Bolling AFB, DC 20332-0001		11. TITLE (Include Security Classification) (See continuation sheet attached)													
12. PERSONAL AUTHOR(S) John R. Wright, Ph.D.															
13a. TYPE OF REPORT Final	13b. TIME COVERED FROM 15 Oct '93 to 14 Feb '95	14. DATE OF REPORT (Yr., Mo., Day) April 12, 1995	15. PAGE COUNT 47												
16. SUPPLEMENTARY NOTATION															
17. COSATI CODES <table border="1"><tr><th>FIELD</th><th>GROUP</th><th>SUB. GR.</th></tr><tr><td></td><td></td><td></td></tr><tr><td></td><td></td><td></td></tr><tr><td></td><td></td><td></td></tr></table>		FIELD	GROUP	SUB. GR.										18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB. GR.													
19. ABSTRACT (Continue on reverse if necessary and identify by block number) <p>The work reported here focused on chemical characterizations of a polymer with interesting luminescent properties, which promises to be useful as an analytical tool in studies of radiofrequency energy deposition in dielectric absorbers. The polymer is formed by diazotizing a mixture of luminol and 3-amino-L-tyrosine and, after removing the aqueous solvent by acetone precipitation, aging the diazotized products for a month at 50 C (i.e., a solid-state reaction). The polymer is anionic and may be precipitated with barium divalent cation. For the purpose of further structural characterizations it was found that this water-soluble polymer may be fragmented by digestion in an aqua regia-like mixture (50:50 concentrated HCl/HNO₃), which converts the broadline hydrogen nmr spectrum typical of polymers to a complex pattern of narrow lines. Capillary electrophoresis revealed approximately 24 anionic products in the mixture with two predominating.</p> <p style="text-align: right;">Continued on attached page.....</p>															
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS <input type="checkbox"/>		21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED													
22a. NAME OF RESPONSIBLE INDIVIDUAL Dr. Walter Kozumbo		22b. TELEPHONE NUMBER (Include Area Code) (202) 767-5021	22c. OFFICE SYMBOL NL												



DTIC QUALITY INSPECTED 6

APR 1995

TITLE: "NMR Characterization of Polymers Formed in Diazotizing Mixtures of Luminol and 3-Amino-L-tyrosine"

ABSTRACT

The neutral forms of these products are soluble in organic solvents, and they are also soluble in water if they are titrated to their anionic forms (pKa about 3.25) using NaOH. Nmr spectroscopy was used to follow fractionations of the fragment mixture on silica gel columns using an elutropic sequence of organic solvents, and at least three significant products were detected, along with numerous lesser components. One of the major products appeared to be a luminol derivative. Ion exchange methods should be applicable to this system, but a working medium has not been found. The best method of separation appears to be a combination of silica gel column chromatography followed by preparative silica gel TLC.

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NMR Characterization of Polymers Formed in Diazotizing
Mixtures of Luminol and 3-Amino-L-tyrosine

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FINAL REPORT

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Date Submitted:	April 12, 1995
Report Due Date:	April 14, 1995 (to AFOSR/NL)
Contract Number:	F49620-94-1-0014

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ACKNOWLEDGEMENTS

The students and the P.I. involved in this research project wish to express their gratitude to the United States Air Force and the Air Force Office of Scientific Research for funding support. We specifically acknowledge Dr. Johnathan Kiel at Brooks AFB and Dr. Walter Kozumbo at Bolling AFB for their help and advice. Input from Dr. Jill Parker and Maj. Eric Holwit at Brooks AFB was also very helpful and is appreciated. Though not funded by AFOSR, Roger McGehee received local support to continue the DALM project under the P.I.'s direction during the summer of 1993, i.e., just before the current funding period started. During that time, Roger found the aqua regia fragmentation method described in this report.

1. INTRODUCTION

This project is focused on a characterization of the structure of a polymer of luminol and 3-amino-L-tyrosine (3-AT). This polymer has been given a descriptive name "diazoluminomelanin" (DALM) since its structure is not known. It possesses both chemiluminescent and melanin-like properties and one of its precursors (3-AT) is structurally similar to the precursor of biological melanin. The chemiluminescent and sonochemiluminescent properties of DALM have been described in an earlier report (1). The chemiluminescence of the DALM anionic polymer may be visualized, for example, by subjecting the polymer to electrophoresis on a cellulose acetate zone electrophoresis strip at pH 6, which causes the brown polymer band to move away from the origin. If the electrophorogram is then blotted with another strip containing alkaline H_2O_2 , the dark adapted eye can detect chemiluminescence at the location of the brown polymer band and also at the origin (probably unreacted luminol). The polymer's chemiluminescence is enhanced by dissolved carbon dioxide. The sonochemiluminescent effects are not as easily reproducible, possibly due to variations in the DALM preparations in spite of attempts to control synthesis conditions (2) or perhaps impurities in the activating substance (potassium formate), but the observed properties are interesting and probably will have practical applications when all of the influencing factors have been identified and controlled, e.g., in microwave dosimetry. Alkaline DALM containing both H_2O_2 and

the carbonate anion produced a bright strobe of chemiluminescence (3) under conditions which did not cause significant heating on the sample when pulsed microwave fluences were applied. This transient phenomenon may have derived from the so-called microwave acoustic effect (4) since a "pop" could be heard when the pulse of microwave was applied. The deposition of a narrow pulse of microwave energy has been shown to induce an acoustic wave, and this could have lead to sonochemiluminescence. Certainly, the pulse energy was sufficient to induce a strong acoustic wave. Various investigators have thought that sonic waves, if they are intense enough, might induce homolytic bond cleavage through large, transient deviations from the apparent temperature, a result of cavitations in the target material (5), thus producing free radicals which could activate any chemiluminescent species present (such as luminol or its derivatives). Solutions of DALM containing luminol and the formate anion have been observed to produce a strong sonochemiluminescence, although the phenomenon has been hard to reproduce, as noted above.

In earlier studies it was found that diazotization of mixtures of luminol and 3-amino-L-tyrosine (3AT) using acidic NaNO_2 in a dimethylsulfoxide/water solvent system produced, initially, a yellow-brown solution containing diazonium derivatives of the two aromatic amines (6). Acetone precipitation of these products was followed by a slow reaction (over a period of weeks) which leads to a brown,

anionic polymer; in other words, most of the polymerization takes place in the precipitated solid rather than the original diazotizing solution. The polymeric substance was given the name diazoluminomelanin (DALM) on the basis of its color and its origin from a substituted tyrosine (7), but its resemblance to a melanin could be superficial since DALM has not been established as a melanin on the basis of molecular structure. For that matter, the biological melanins are structurally diverse, and structural characterizations of melanins are incomplete to this day (8). The pressing concern is thus a characterization of the chemical structure of DALM, so that its further study and development may proceed in a more rational manner.

A similar polymerization was observed for the diazotization product of 3AT alone (hereafter DAT for diazotized 3AT), which also produces a brown, anionic polymer under a variety of reaction conditions, including a slow reaction in the original diazotizing solution. Both polymers, DALM and DAT, exhibited typically broadened nmr spectra.

2. OBJECTIVES

The main objective of this project is a structural characterization of the polymer formed in diazotized mixtures of 3-AT and luminol (DALM). This objective was agreed upon with Dr. Johnathan Kiel during a 1992 coordination visit at the sponsoring laboratory at Brooks AFB, Texas. It was recommended that future studies be focused almost entirely on

a structural characterization of the DALM and DAT polymers since a better understanding of their interesting properties will depend upon structural insight. A proportionately smaller effort should be devoted to controlled experiments which better define the sonochemiluminescent properties of DALM.

3. EXPERIMENTAL METHODS

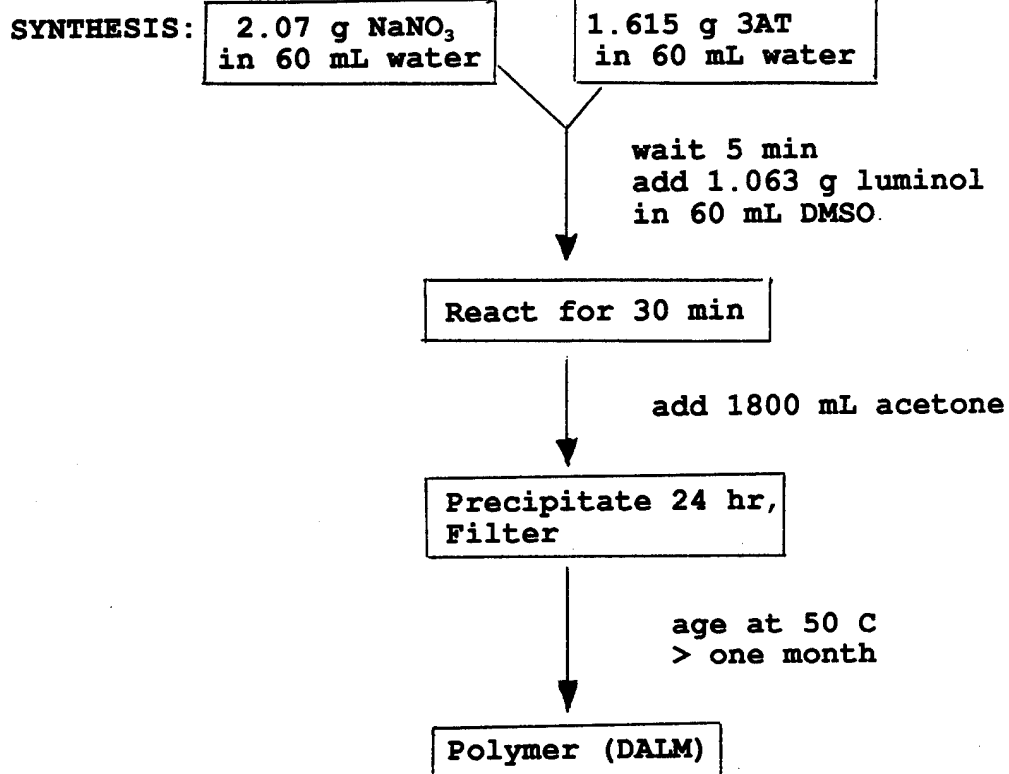
3.1. Materials

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), sodium nitrite and 3-amino-L-tyrosine were obtained from Sigma Chemicals, and potassium formate was purchased from Aldrich Chemicals (stock number 29,445-4). Other substances used in the investigation were obtained from Sigma, Aldrich or Fluka.

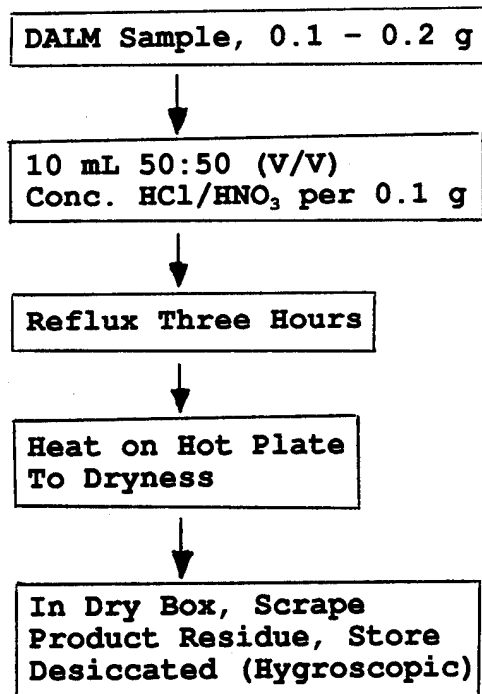
3.2. Preparation of the DALM Polymer

The synthesis protocol for DALM, as it was used here, is presented in Fig. 1. In previous work (6) it was shown by means of differential scanning calorimetry that the exotherm due to unreacted diazonium groups fades away over a period of weeks when the DALM-forming mixture is aged at room temperature, and this decomposition reaction corresponded to the onset of polymer formation. Decomposition of the diazonium species may also be followed by means of nmr spectroscopy, i.e., if samples of the polymer-forming solid are examined as a function of aging time, the narrow

Figure 1 - DALM Synthesis and Fragmentation Protocols



FRAGMENTATION:



resonances of the diazonium species give way to the broad polymer resonances.

The DALM polymer used in the experiments reported here was selected from preparations which had aged one month at 50 C in a drying oven to accelerate the rate of polymerization.

3.3. Precipitation of the polymer by Ba^{2+} ion

The mature DALM polymer is anionic, and since the acetone precipitated solid is a heterogeneous mixture containing both sodium nitrite and the DALM precursor solid, some thought was given to a method for removing nitrite ion present. The polymer precipitates in the presence of barium divalent cation, while the solubility of barium nitrite is about 63 g/100 mL at 20 C. If oxidation of nitrite occurs, the solubility of barium nitrate is 8.7 g/100 mL at 20 C (9). Dilute (0.1 g/100 mL) solutions of aged DALM preparations are thus precipitated with barium ion and washed five times by centrifuging and resuspending the dark brown sediment in pure water. The counterion barium is then exchanged with sodium using an equivalent amount of sodium Chelex-100 resin; the Chelex exchange procedure restores the solubility of the polymer. The dark brown polymer solution may then be evaporated and the residue desiccated over phosphorous pentoxide. A dried sample obtained in this manner was submitted to Oneida Research Services for an elemental analysis, which accounted for all of the elements possibly present, even from solvents.

This precipitation followed by ion exchange chromatography was considered as a possible way to purify the DALM, but its use is limited in view of the residual of barium counterion, which cannot be removed.

3.4. Polymer fragmentation with HCl/HNO_3

Samples of the mature DALM polymer, 0.1 to 0.2 g, are suspended in 10 mL of a 50/50 (vol/vol) mixture of concentrated hydrochloric and nitric acids. This is a kind of aqua regia, although it is noted that literature values for aqua regia vary (10); usually the HCl/HNO_3 volume ratio is taken as 3/1. The mixture is refluxed with stirring for three hours, during which time the dark brown pigmentation of DALM is observed to bleach. The final digest solution is yellow colored. This product is then poured into a Petri dish and evaporated to dryness on a hot plate. The residue, which is hygroscopic, is scraped out and stored desiccated over phosphorous pentoxide.

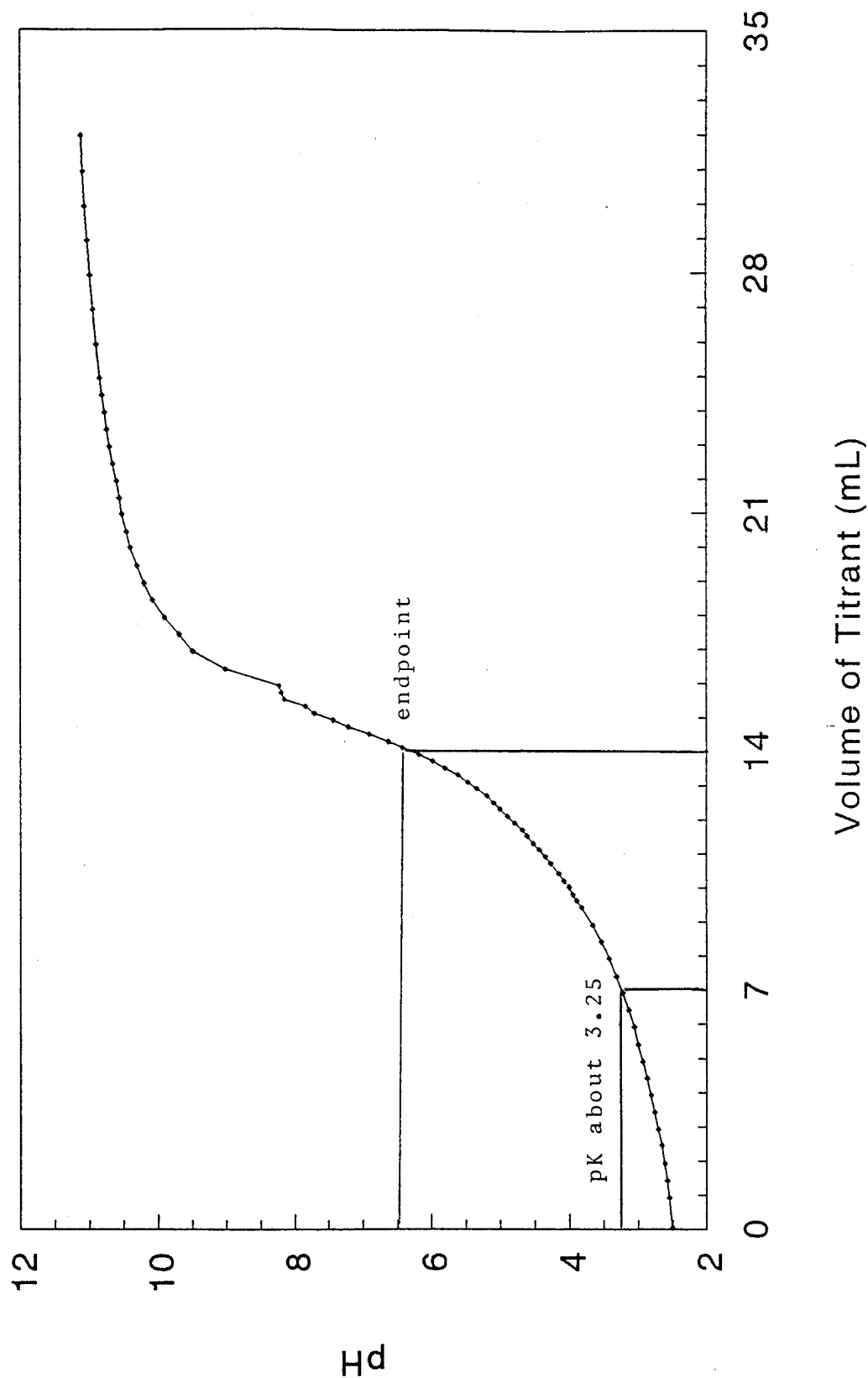
3.5. Titrations of the polymer fragment mixture

The residue remaining after evaporation of the HCl/HNO_3 -treated DALM samples was dried, weighed by difference and titrated against 0.0144 N NaOH, recording equivalents added versus pH readings from a calibrated Fisher Acumet 925 pH meter. A sample titration curve is shown in Fig. 2.

Figure 2 - Titration of Aqua Regia Fragmentation Products of the
DALM Polymer

.0144 M NaOH (titrant)

—◆— fle: kw31695



3.6. Electrophoretic separations

A capillary electrophorogram of anionic components of the DALM fragments is shown in Fig. 3 along with the conditions of the separation. The electrophorogram was obtained at Oklahoma State University by Dr. Tim Smith using a capillary instrument (Dr. Smith was an earlier undergraduate participant in the ongoing AFOSR project, and he is now at Monsanto in St. Louis, Missouri).

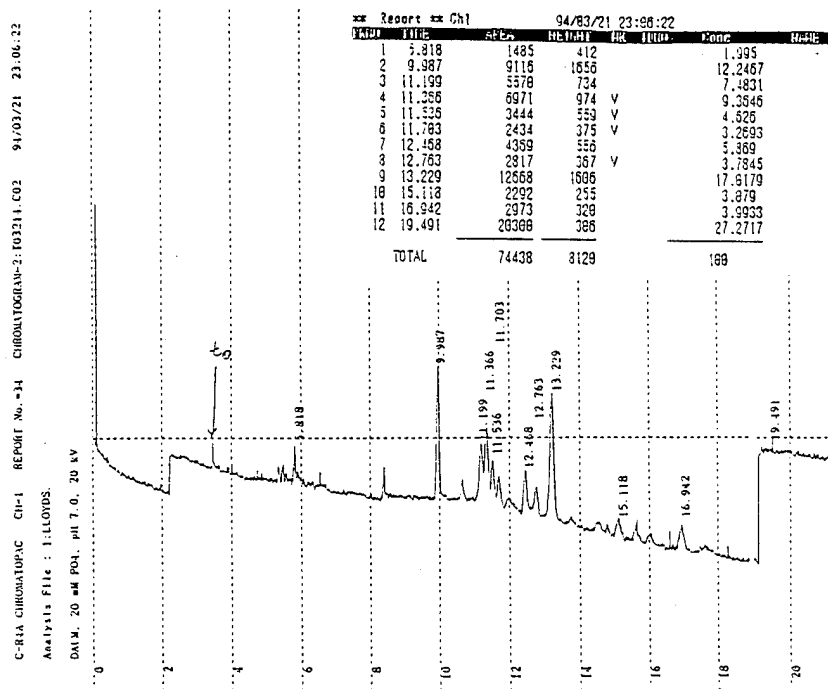
The fragment mixture may also be subjected to separations on Sepraphore III zone electrophoresis strips in pH 8.8 barbital buffer, but this method reveals only three poorly resolved bands visually and the same under longwave UV light.

3.7. Thin layer chromatography separations

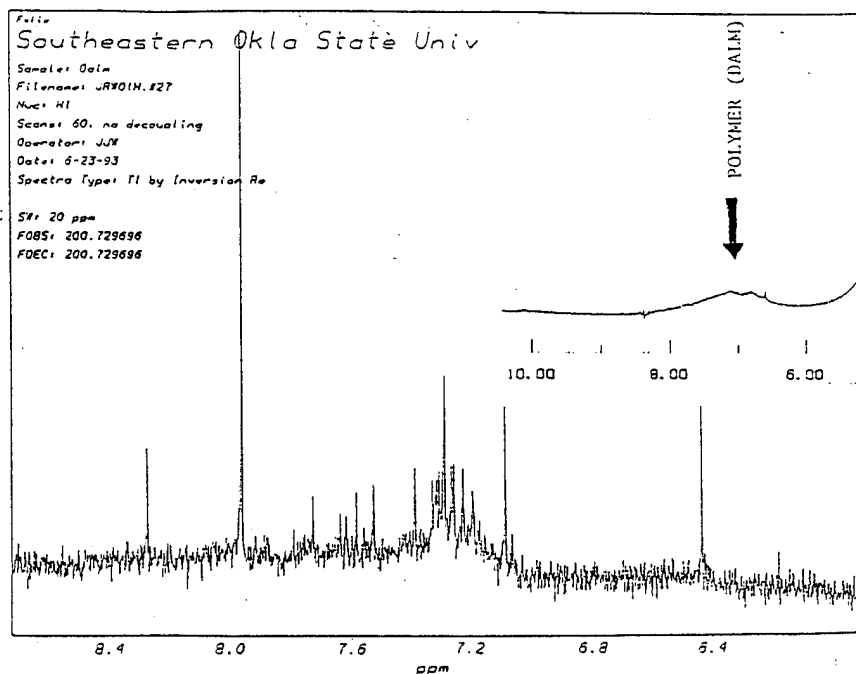
Neutralized DALM fragments ($\text{pH} < 3$) may be separated on silica gel TLC plates (Alltech silica gel 60 F-254, 5 x 20 cm, layer thickness 0.250 mm). Pure solvents and solvent mixtures in the elutropic range toluene \rightarrow dioxane \rightarrow acetone \rightarrow methanol were found to be appropriate for these substances. Components may be visualized by UV fluorescence, but it is noted that UV bands can be deceptive in that a significant component may be overlooked if it is not fluorescence active. For this reason, some of the chromatograms were visualized in iodine vapor.

Figure 3 - Capillary Electrophoresis and Nmr of the DAIM
Fragment Mixture (from aqua regia)

A - Capillary Electrophorogram of Fragments



B - Proton Nmr of the Fragment Mixture



3.8. Silica gel column separations

In the earlier work, separations of fragmented DALM were carried out on 2 cm diameter x 40 cm columns of Malinckrodt Silicar, 60-200 mesh, eluting with stepped gradients of the solvent series described in part 3.7., above. The Silicar is activated at 105 C overnight before preparing columns. In the last two months of the effort, columns with a more homogeneous particle size distribution were prepared using Fisher 60-100 mesh chromatographic silica gel (S735-1), activated as described above. The latter columns measured 4.2 cm diameter by 12 cm deep in these preliminary studies.

3.9. Other separation attempts

Products of the aqua regia digestion are anionic and water soluble above pH 5, and in view of the success of capillary electrophoresis in resolving the mixture of DALM fragments, attempts were made to effect a separation of the mixture on ion exchange columns, using either pH gradient or ionic strength gradient elutions. The stationary phases tried were DEAE Sephadex (50-120 mesh), Amberlite IR-120 resin (20-50 mesh) and Amberlyst-15 resin (16-50 mesh). None of these produced well-resolved separations, and the colored material adhered tenaciously to the columns.

3.10. Nmr Measurements

Nmr spectra were obtained by means of a 4.7 Tesla Chemagnetics A200 liquids system which has a quadrature

detection receiver and which has been developed for various 2D applications. The available 2D data acquisition pulse and phasing programs include H,H-COSY, H,C-COSY (HETCOR), J-resolved hydrogen spectroscopy, MKE spectroscopy. The system was extensively upgraded in November 1994 (USAF/AFOSR funds were not involved). Other versions of 2D nmr are easily added since the instrument's operating system includes developmental software. The instrument accomodates a 1 cm diameter cuvette, and its X-probe is broadband and tunes the range from nitrogen to phosphorous, which includes carbon.

The ion-exchange and silica gel column separations described in this report were monitored by ^1H -nmr spectroscopy. The general procedure involves evaporating fractions to dryness, then dissolving the residue in alkaline D_2O for spectroscopy.

3.11. Computations and Data Analysis

Two dimensional nmr spectra were processed using FELIX software and an ALR 80386/387 system with a 120 MB hard disk and a 16 MB RAM drive. Various computations and data presentations were accomplished with an IBM 466 DX/2D, using a variety of licensed programs (e.g., SlideWrite, Mathcad, Huckel MO Calculator, several statistical analysis programs, etc.).

methods for resolving the polymer fragments have been identified, and at this writing the separation methods are being developed, refined and scaled up. Both adsorption chromatography and capillary electrophoresis of the aqua regia digests reveal two major fragments along with numerous lesser products. The work has thus been making good progress.

4.1. Precipitation of the DALM polymer by Ba^{2+} cation

The method described in 3.3. was used in an attempt to isolate the anionic DALM polymer as a sodium salt, and the elemental analysis presented in Table I was obtained:

TABLE I: Elemental Analysis of Ba^{2+} Precipitated, Na^+ Exchanged DALM

Found:	carbon	29.29%
	hydrogen	3.34%
	nitrogen	8.19%
	oxygen	37.21% (by difference)
	sodium	11.02%
	barium	9.19%
	sulfur	1.76%

It is noted that dimethyl sulfoxide is difficult to remove from DALM preparations, and the ^1H -nmr resonance persists in the product, even after a thorough acetone washing of the dried material. Nevertheless, some caution is warranted since

DMSO may have actually entered the reaction. It is also seen that some of the precipitating barium ion is tenaciously bound, and the exchange with sodium ion on Chelex-100 is only partial (for this reason, the barium-precipitation method is not used to prepare DALM for structural studies). The other elements are expected from the known starting materials.

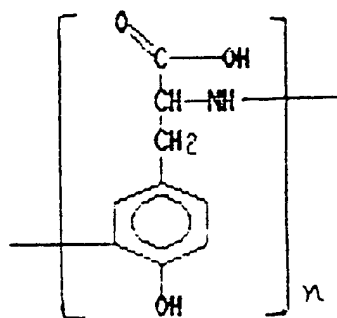
The element molar ratios of sodium/barium DALM are compared in Table II with theoretical values for 3-AT and luminol:

TABLE II: A Comparison of Element Molar Ratios of DALM with Those of 3-AT and Luminol

RATIO	DALM	3-AT	LUMINOL
H/C	1.36	1.33	0.88
C/N	4.17	4.50	2.67
C/O	1.04	3.00	4.00
C/Na	5.09	(9.00)	-
C/Ba	36.4	-	-
C/S	44.4	-	-

One should not read too much into the C/O ratio since it is possible that hydration water remains in the material in spite of its being dried over the strong desiccant, phosphorous pentoxide. Nevertheless, with regard to luminol, a C/O ratio closer to that observed in DALM would result if N₂ were lost from the luminol heterocycle and the carbonyls became

carboxylic acid groups. The H/C and C/N (but not C/O) ratios of DALM are similar to those of 3-AT and a possible arylamine linkage considered in an earlier report (11) and shown here could be involved:



This type of structure requires one Na^+ counterion per 3-AT unit for a C/Na ratio of 9 (the parenthetical entry under 3-AT in Table II), whereas a ratio of about 5 is observed. If luminol incorporates into the polymer as a pendant group and with a loss of at least some of the nitrogen heterocycles, a ratio smaller than 5 is possible. It is noted that titration data for DALM fragments (see 4.2. below) are more consistent with a larger mass per carboxylate group, but the fragmentation process may have removed some of the carboxylate groups. The P.I. is skeptical of the structure shown above, for one thing because decolorization occurs when DALM is digested in an aqua regia-like mixture of HCl and HNO_3 , indicating that the structure probably has conjugated double

bonds.

4.2. A method for fragmenting DALM.

Digestion of DALM according to the method described in 3.4. yields a complex mixture of products. A ^1H -nmr spectrum of the aromatic region of this material in comparison with the broad polymer resonance is shown in Fig. 3b, which reveals numerous lines suggestive of a mixture. It is noted that the pattern of narrow aromatic nmr lines observed in the fragmentation product approximates the broad envelope of the high molecular weight polymer itself, and it is probable that these protons originated in the aromatic parts of the starting materials, i.e., luminol and 3-AT. There are no spectral signatures identical in chemical shift to those of the starting materials.

A capillary electrophorogram of anions present in aqua regia digested DALM is presented in Fig. 3a, and the electrophorogram reveals at least two dozen components, with two species (at 9.99 and 13.23 minutes) predominating. This gives some feel for the task ahead in separating the major components of the fragmentation mixture.

The residue obtained on evaporating HCl and HNO_3 after an aqua regia digestion of DALM (see 3.4.) is not soluble in water, but rather in an organic solvent such as dioxane. However, the residue is soluble in alkaline water, going into solution near pH 5 when it is titrated with aqueous NaOH . A pH titration curve for the mixture is shown in Fig. 2, and the

half equivalence point occurs near pH 3.25 (where solubility is noticable), although some variability is seen between preparations. This result is consistent with the pK_a range of carboxylate groups, especially those attached to nitrated aromatic structures or a halogenated aliphatic group. It is also consistent with the anionic components observed during capillary electrophoresis of the mixture, as shown in Fig. 3a. The presence of carboxylate groups would not be surprising from a consideration of the starting materials since 3-AT is a carboxylic acid. Also, the loss of two nitrogens from the luminol heterocycle ring would yield aminophthalic acid or perhaps a derivative of aminophthalic acid in the case of DALM's structure. Based on a weighed sample of DALM fragments and the volume of NaOH added to reach the endpoint, the apparent equivalent mass of the fragment mixture is found to be 258 g/mol H^+ . Although this value cannot be attributed to a single substance, it is within a 50 % excess of the molecular masses of 3-AT (196 g/mol) and luminol (177 g/mol). DALM itself migrates as an anion on electrophoresis strips and is precipitated by Ba^{2+} cation, as described in 4.1., and the fragments clearly have ionophoric groups, consistent with the properties of solubility in low polarity solvents at low pH and aqueous solubility at neutral and higher pH values.

Three different ion exchange media have been tried in an effort to resolve fragmentation components at pH values above 5, using both pH and ionic strength gradients, with generally poor results (see 3.9.). In view of the success of capillary

electrophoresis in resolving fragmentation components, the possibility of finding an appropriate ion exchange method should still be regarded as open.

Thin Layer Chromatography (TLC) on silical gel using pure and mixed solvents in the range 50/50 toluene/dioxane -> dioxane -> acetone -> methanol produced generally good resolution of the fragmentation components, and sketches of examples of fluorescence-quenched TLC plates are shown in Fig. 4. The progression in Fig. 4 of the results for five different solvents used just the crude aqua regia fragmentation product mixture. The example on the continuation page of Fig. 4 presenting a TLC analysis of a silica gel column fraction clearly shows the latter to be a mixture rather than a pure compound. Separations on TLC plates requires applying DALM fragments in their neutral form, i.e., the residue is acidified and extracted into an organic solvent such as dioxane.

Success with silica gel TLC implies that HPLC on a silica gel column should produce good resolution of the components. The department recently purchased a new HPLC instrument, but it developed trouble (in another faculty member's project) and was not accessible to us most of the time due to its problems. During a period when the instrument was in working order we tried to carry out separations of fragmentation products on a 4.6 mm I.D. x 15 cm length column of Alltech 86-100-D5 Microsorb-MV silica gel using either pure dioxane or mixed solvents as the eluent. Some narrow bands were seen to elute

Figure 4 - Silica Gel TLC of Neutral DAIM Fragments in a Progression of Solvents

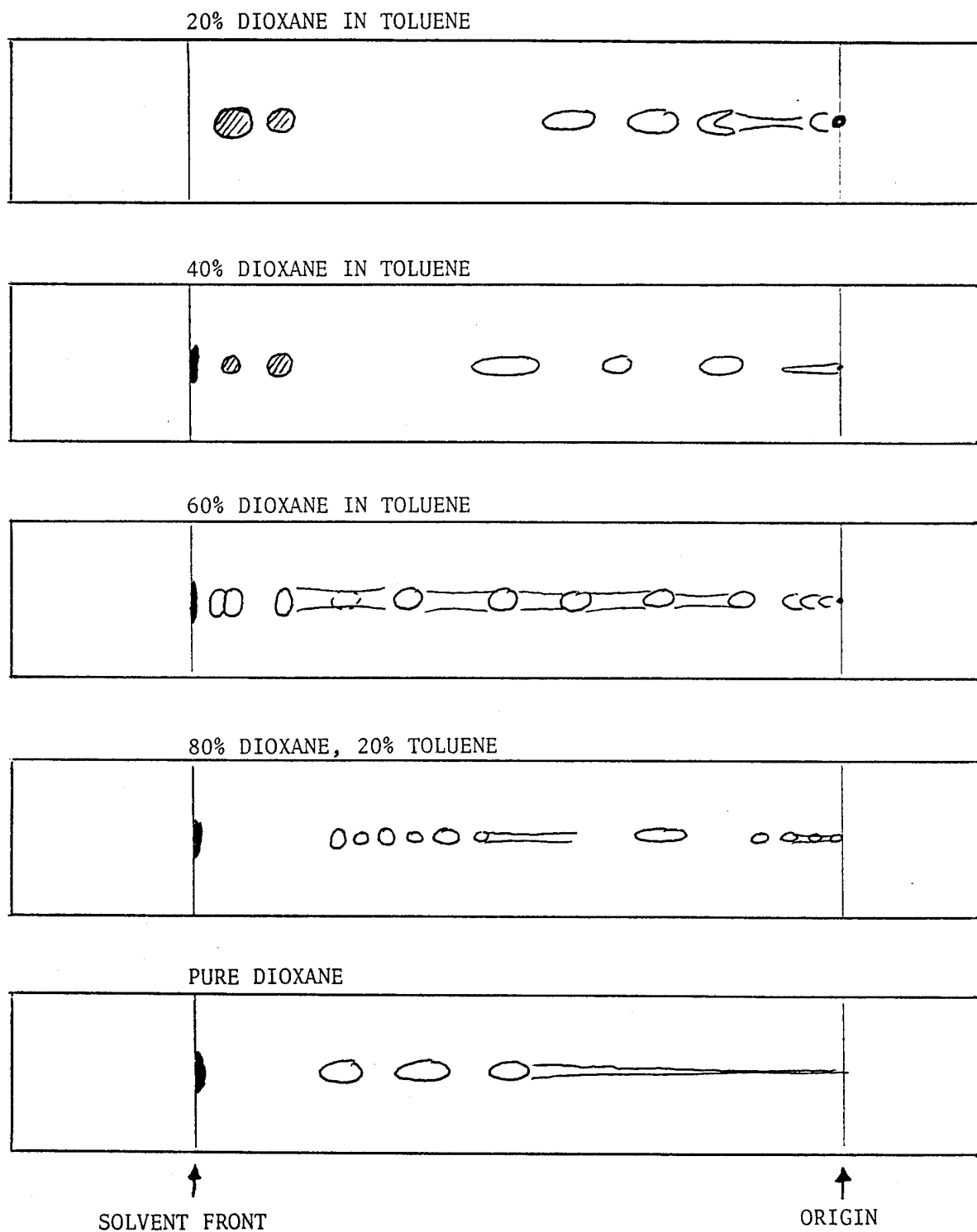
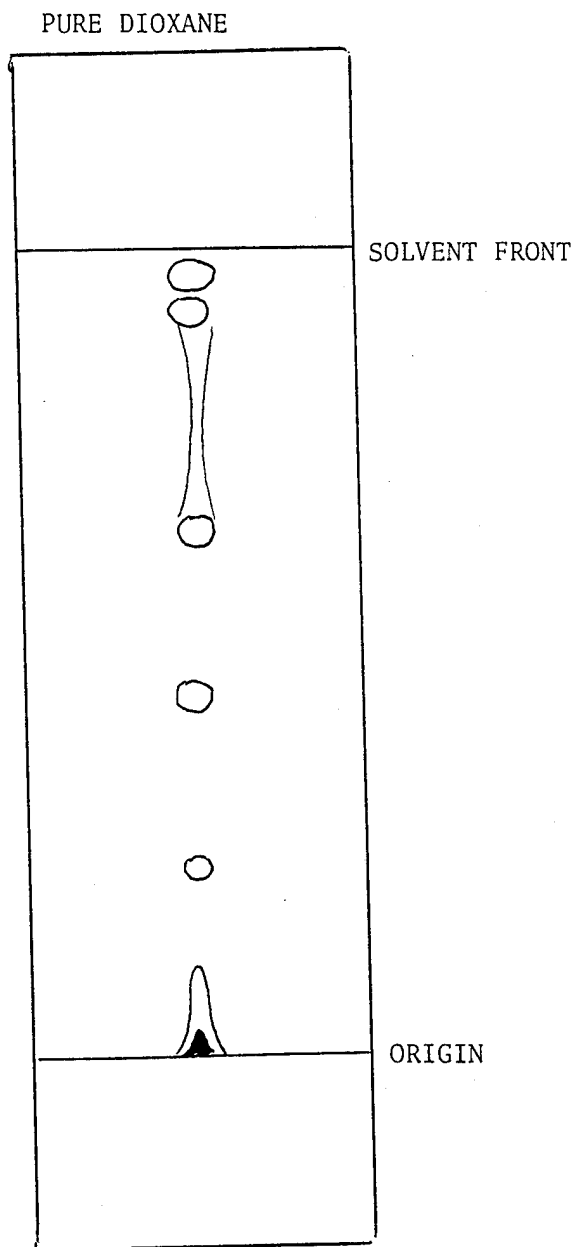


Figure 4 - Continued - TLC of the Material Used To Obtain the Proton Nmr Spectrum of Fig. 6e. (this is a fraction from a silica gel column and obviously still a mixture)



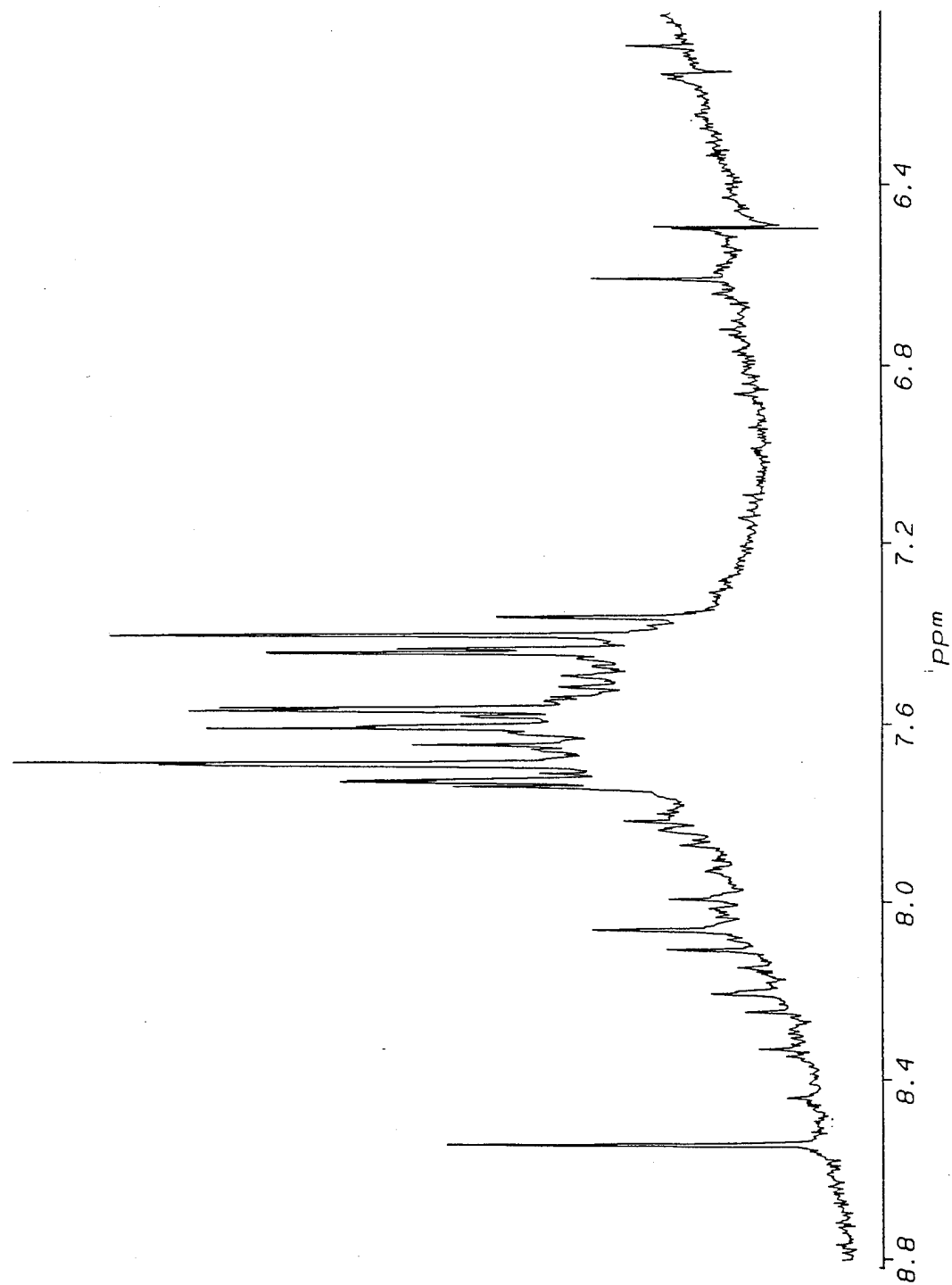
from the column, but there were problems with residual material, probably polymeric, hanging up in the column and then bleeding off slowly. This kind of result is consistent with what is seen Fig. 5 (discussed in the following paragraph), and the group at Brooks AFB encountered similar problems in earlier attempts on DALM using HPLC.

The success of fragmentation studies will depend on isolating species which have at least two monomeric units and an unaltered polymeric linkage. One might expect an optimum digestion time, where fragmentation has not quite produced a total destruction of the polymer but where the product mixture is not excessively complex. Fig. 5 shows the effect of a short digestion time (one hour instead of three) on the nmr spectrum of the total product mixture. In this figure narrow lines superimpose on a broader, unresolved, polymer-like envelope. Figure 5 should be compared with Fig. 3b; it is between the cases of total fragmentation and the that of the original polymer. The residual polymer component appears to be the agent which compromises HPLC columns, as described above.

Variability between polymer digestions may be due to the relative sizes of DALM particles exposed to the aqua regia mixture, i.e., larger particles require more time for decomposition. For this reason the material should be ground to fine particles before the digestion, and the reaction mixture should be stirred thoroughly.

Nmr spectra such as those of Fig. 3b and those of Fig. 6

Figure 5 - Nmr Spectra Showing the Effect of a Short Digestion Time on DALM Fragments



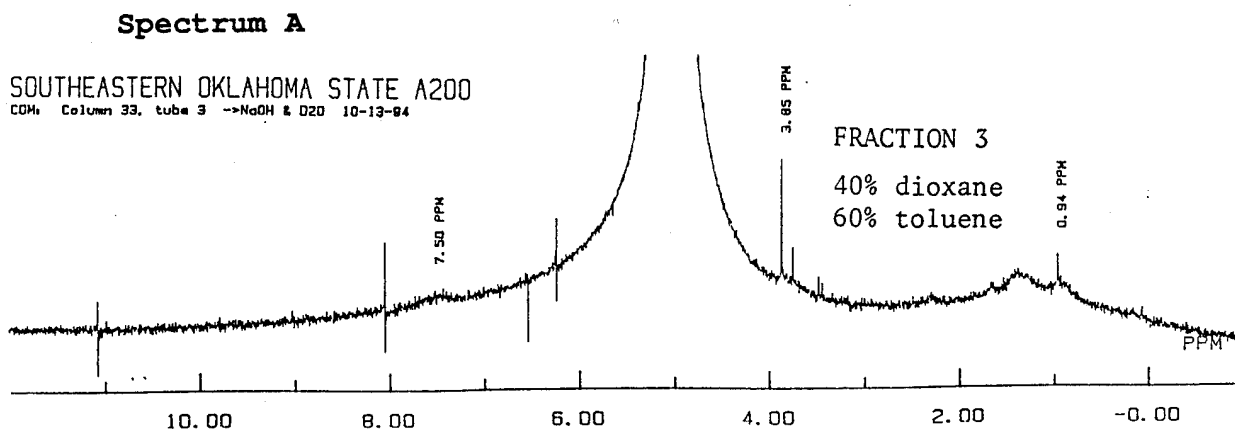
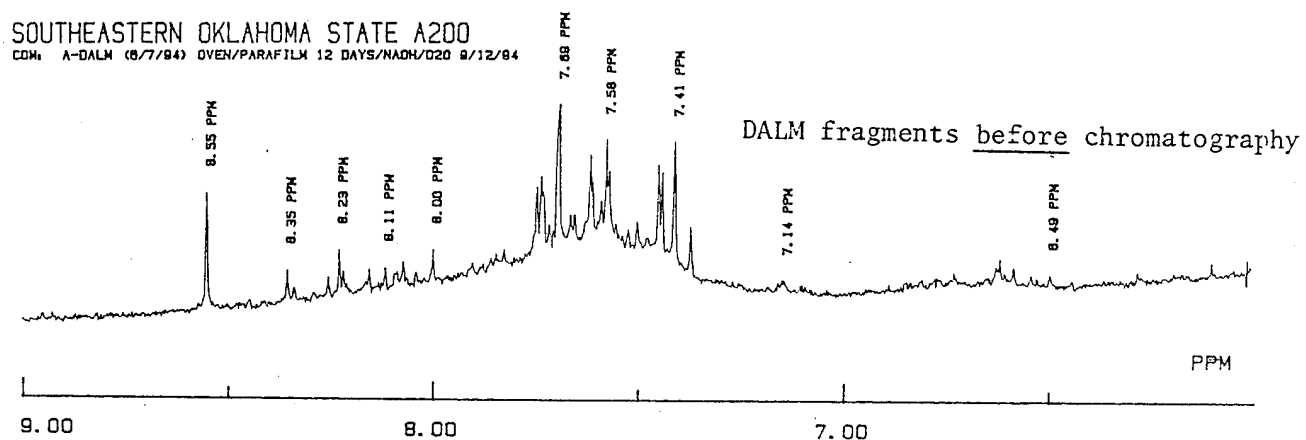
were obtained for preparations which involved minimal delay (24 - 48 hours) after the aqua regia step. It was noted, however, that if the hygroscopic fragment mixture was allowed to become wet in room air and allowed to stand for a week, nmr spectra began to show the broadened lines of a polymer. Thus, the fragments are considered reactive and unstable and should be handled accordingly, minimizing time to complete experiments.

4.3. Separation of DALM fragments on columns of silica gel and by preparative TLC

The success of TLC in resolving components of aqua regia fragmented DALM led to attempts to separate ponderable amounts of the neutral fragments (low pH) on medium sized columns of silica gel (see 3.8.). Enriched components eluting from these columns were transferred to alkaline D₂O and examined by ¹H-nmr spectroscopy. This was judged to be a better approach than following UV/visible absorbance because of limitations imposed by solvent absorption and potentially large variations of optical absorbance between different compounds and because nmr detects the dominant components of a given fraction. The following example is representative of fractionations on a silica gel column. Nmr spectra of these fractions are collected in Fig. 6.

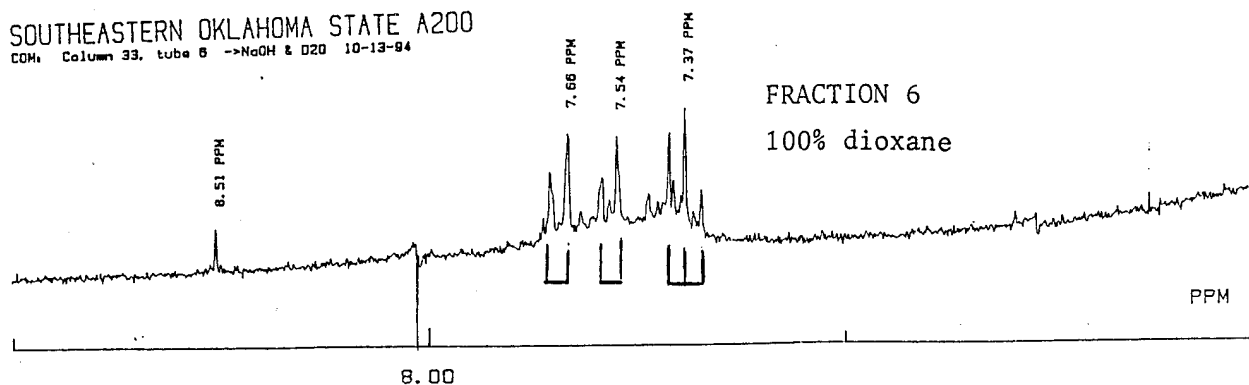
Pooled fraction 3 (40% dioxane/60% toluene) from column 33 apparently contains a minimal amount of material, possibly

Figure 6 - Nmr Spectra of Fractions Eluting From Silica Gel Columns



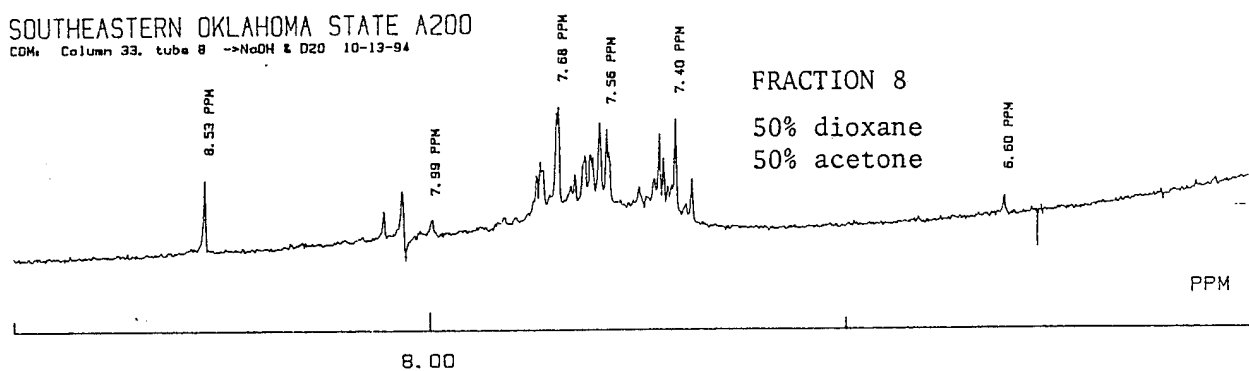
Spectrum B

SOUTHEASTERN OKLAHOMA STATE A200
CDH, Column 33, tube 8 ->NaOH & D2O 10-13-94



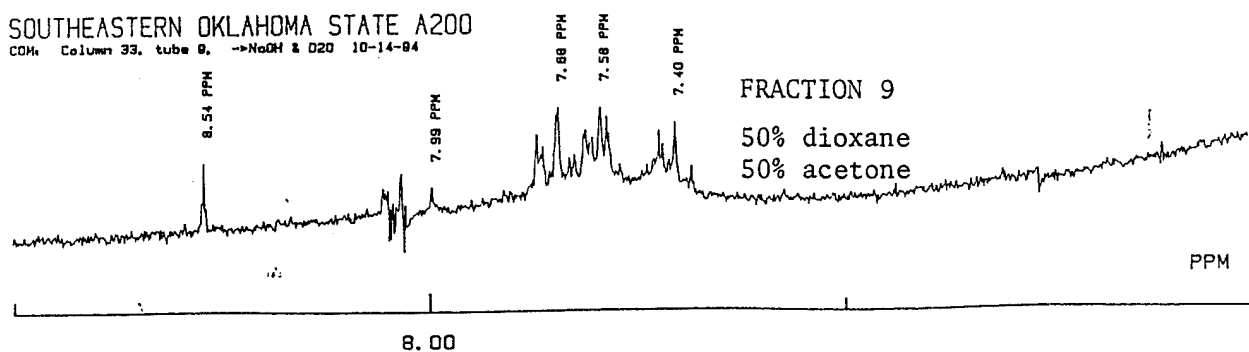
Spectrum C

SOUTHEASTERN OKLAHOMA STATE A200
CDH, Column 33, tube 8 ->NaOH & D2O 10-13-94



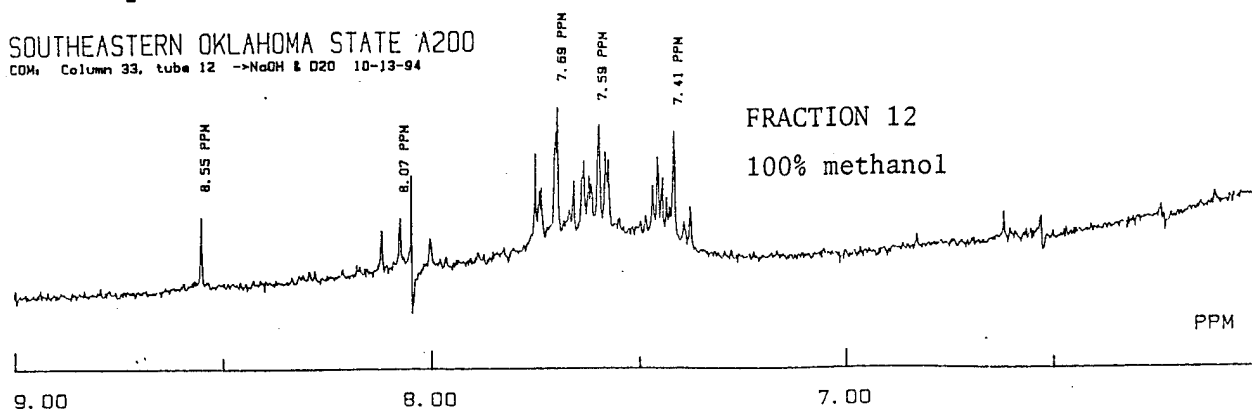
Spectrum D

SOUTHEASTERN OKLAHOMA STATE A200
CDH, Column 33, tube 8, ->NaOH & D2O 10-14-94



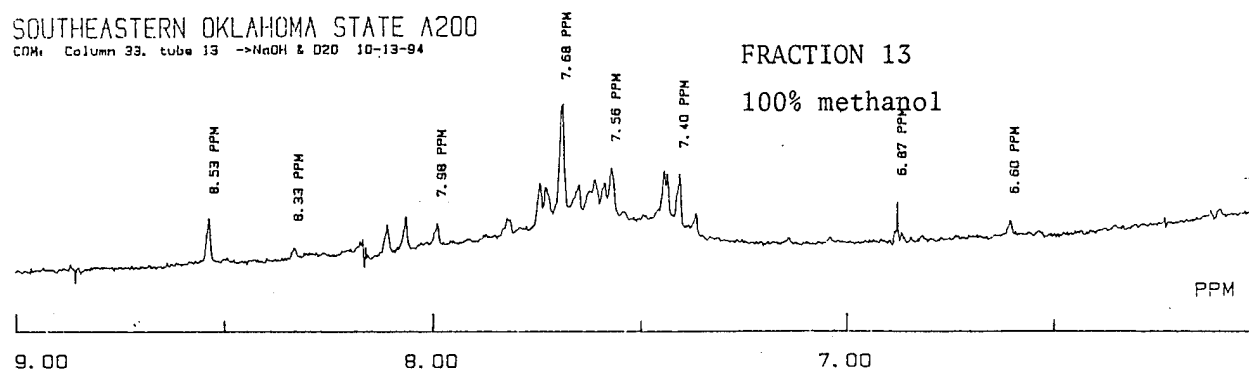
Spectrum E

SOUTHEASTERN OKLAHOMA STATE A200
COM: Column 33, tube 12 ->NaOH & D2O 10-13-94



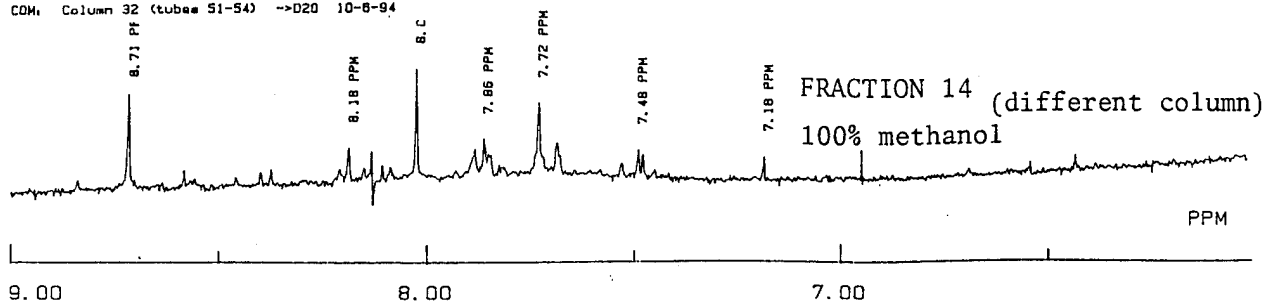
Spectrum F

SOUTHEASTERN OKLAHOMA STATE A200
COM: Column 33, tube 13 ->NaOH & D2O 10-13-94



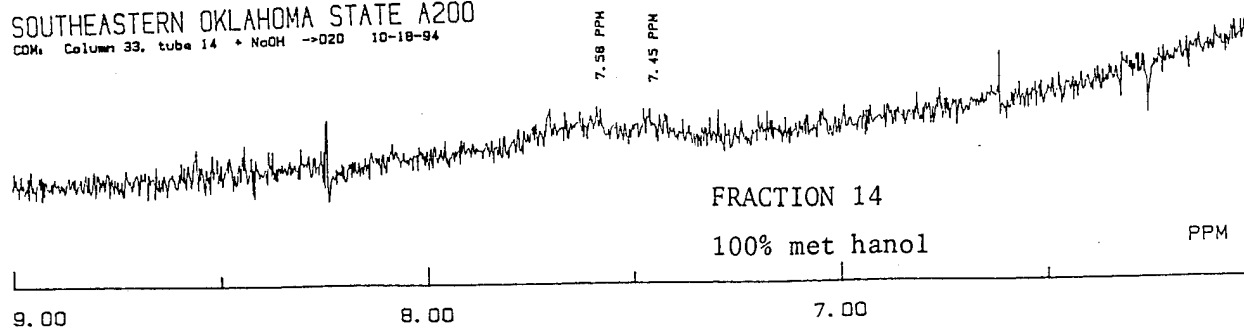
Spectrum G

SOUTHEASTERN OKLAHOMA STATE A200
COM: Column 32 (tubes 51-54) ->D2O 10-8-94



Spectrum H

SOUTHEASTERN OKLAHOMA STATE A200
COM: Column 33, tube 14 + NaOH ->020 10-18-94



polymeric judging from the broad aromatic and aliphatic resonances, or perhaps a complex mixture with many lines overlapping.

The pooled fraction 6 (100% dioxane) from column 33, which measured 2.5 cm diameter by 30 cm, presents a distinct doublet-doublet-triplet signature in the aromatic region (Fig. 6b), and is almost certainly a fragment originating in luminol. The spectrum includes other resonances, and the substance responsible for the signature is only partially resolved from impurities. Crosspeaks in the H,H-COSY spectrum of the DALM fragments (Fig. 7) show clearly that this signature is an actual coupling pattern of three adjacent ring protons (as in luminol), and the coupling constants are quantitatively consistent. Both HMO calculations of charge density and comparisons of the spectra of known compounds related to luminol lead to the observation that if the two doublets are downfield of the triplet, the group at ring position 5 of the luminol moiety is not electron donating, e.g., a group like $-N_2^+$ or $-NO_2$, but if the doublets are upfield with respect to the triplet, the group at position 5 is an electron donor, e.g., a group such as $-NH_2$ or $-OH$. The fragmentation component thus appears to be of the former type.

The proportion of the Fig. 6b fragment has certainly varied between preparations. Compare Fig. 8 with Fig 6b, where the pattern is qualitatively the same but quantitative differences in the impurities are visible.

It would not be surprising to find that the substance of

Figure 7 - Low Resolution H,H-COSY Spectrum of DALM Fragments

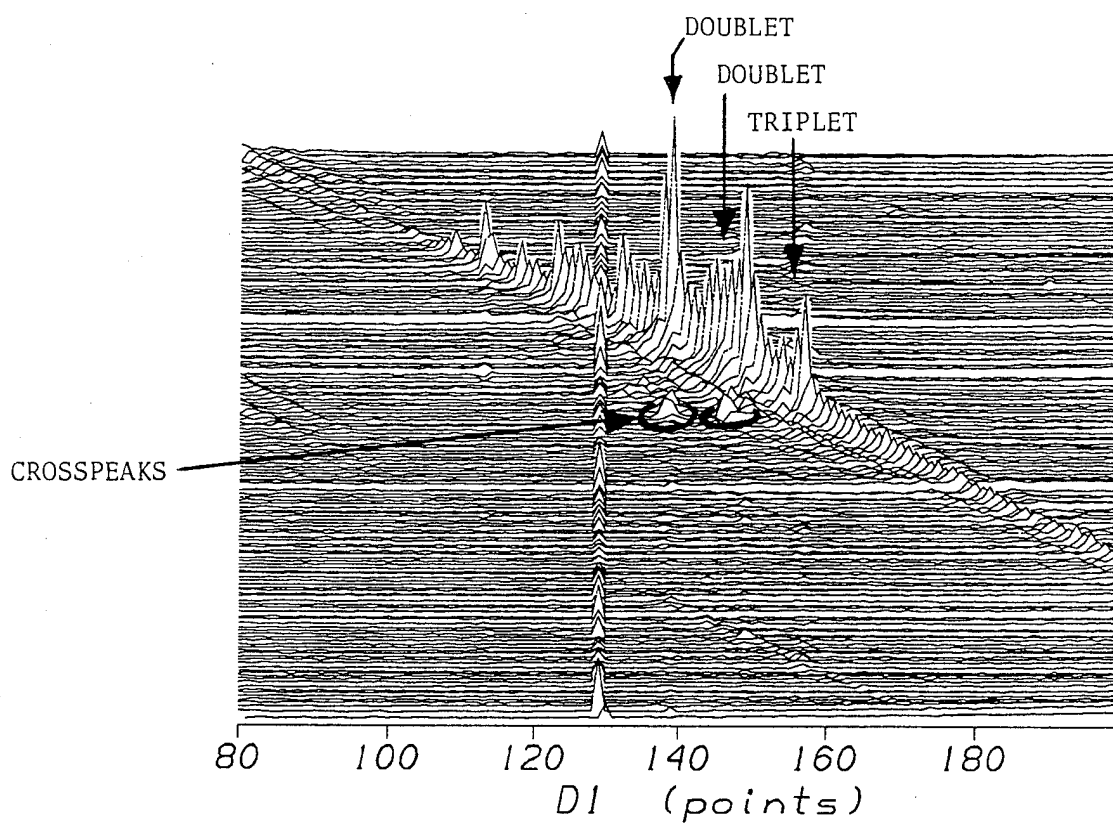


Figure 8 - Spectrum of the Putative Luminol Derivative From
a Larger Scale Preparation (compare with Fig. 6b)

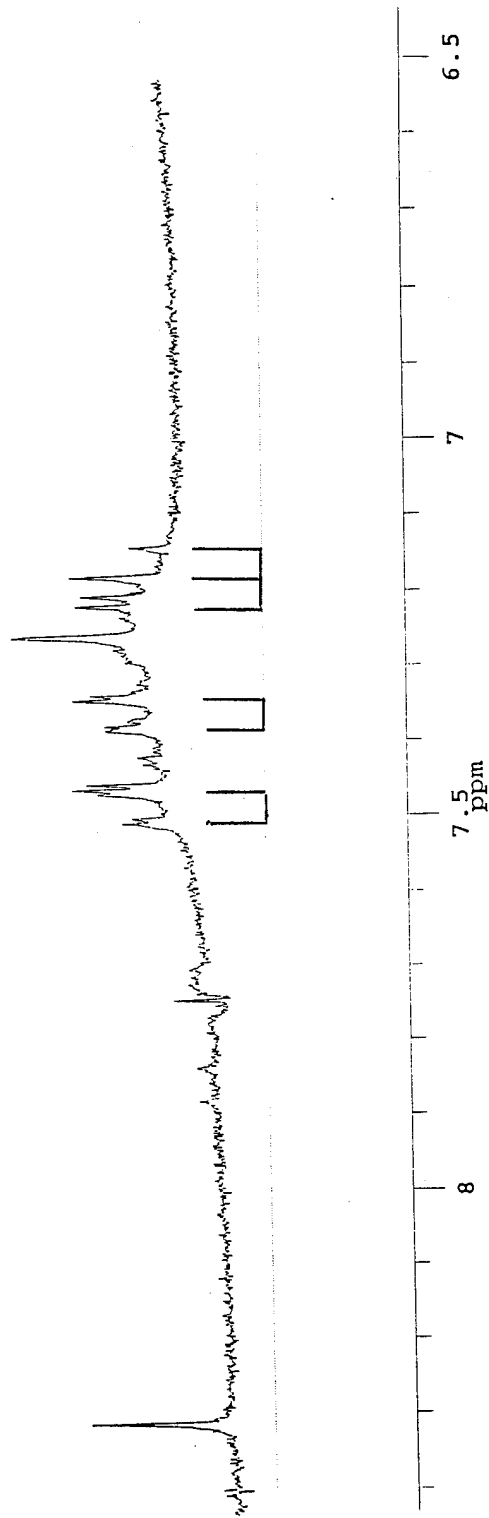


Fig. 6b is a derivative of luminol with a nitrate or nitrosyl group at position 5, considering the nature of the digesting reagent. Comparisons with known substances, i.e., by adding knowns to the solution of this fragment or by noting markedly different spectroscopic properties, show clearly that the substance is not luminol, aminophthalic acid or luminol diazonium. This substance could have originated in the DALM polymer, possibly from a luminol pendant group. Heretofore, there had been some question as to whether or not luminol was actually incorporated into the polymer.

It could be argued that since the crude DALM preparations are known to contain a small amount of unreacted luminol, perhaps this component originated directly from luminol and not from the DALM polymer. But the amount of unreacted luminol is small relative to the polymer and the component of Fig. 6b. Alkaline solutions of DALM do not show a superimposed, prominent signature of low molecular weight luminol (see the inset of Fig 3b).

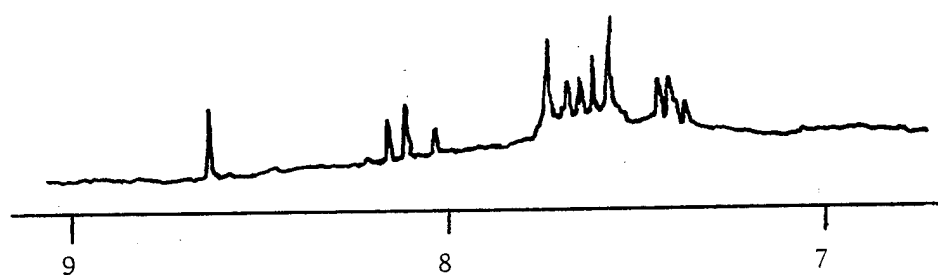
It was noted in the early studies at Brooks AFB (12) that zone electrophorograms of the immediate product of a luminol/3-AT diazotization (i.e., within 30 minutes of the start of the reaction) revealed a brown anionic component which was not seen if the diazotization involved either luminol or 3-AT alone. This is shown in Fig. 10, which is taken from reference 12. The unique component, labeled A, is not DALM but probably an intermediate complex of diazotized forms of 3-AT and luminol which react further to produce DALM

in a slow reaction. Such immediate product mixtures also show more dark brown material at the origin, which may contain additional diazotized luminol and 3-AT in a complex of some type or in an insoluble form. The evidence for a luminol structural element in the polymer is thus strong.

The results of further elution of the column with increasingly polar solvents are shown in Fig. 6c through 6f, which may be compared with the total (soluble) fragmentation product in Figs. 3 and 5. The doublet-doublet-triplet signature tails into these fractions (numbers 8 - 13; the signature is also prominent in the unfractionated product from HCl/HNO₃ digestion), but new resonances appear enhanced near 7.5 ppm (marked by arrows) and downfield of 8 ppm (also marked by arrows). These resonances indicate at least one additional major substance (and possibly more) in pooled fractions 8 - 12. If the doublet-doublet-triplet signature is subtracted from this spectrum, the result is in good agreement with parts of a spectrum obtained from a distinct band eluting from a large silica gel column under similar conditions (only a minimal amount of a dioxane extract of the DALM fragments was applied to the column to avoid an overloaded condition). The spectrum of the latter substance along with the difference spectrum derived from pooled fractions 8 - 12 is shown in Fig. 9; the regions between 7 and 8 ppm are comparable, as is also a singlet at 8.27 ppm. However, the three lines immediately downfield of 8 ppm are not seen, indicating that they belong to a third, lesser substance.

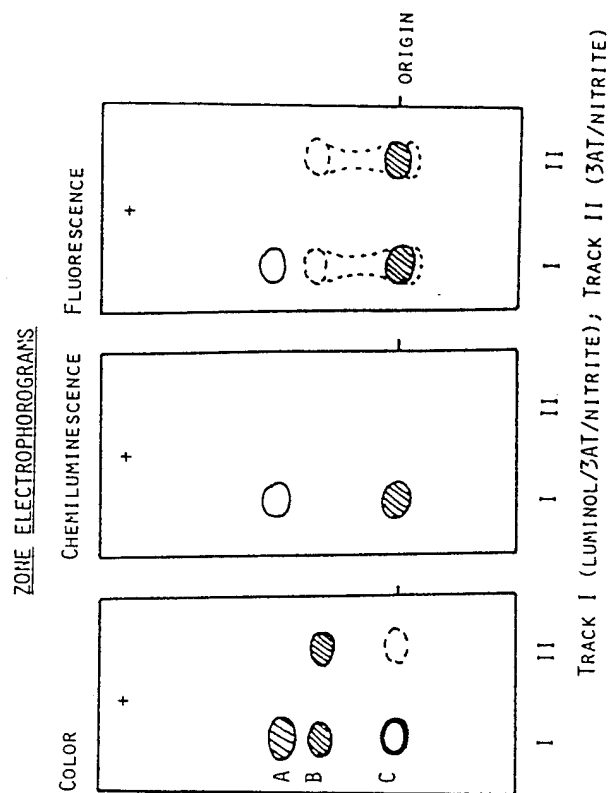
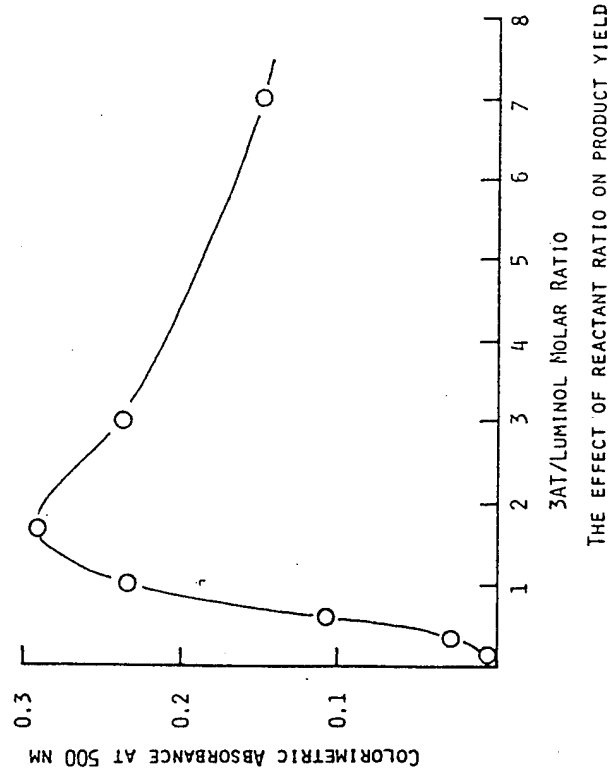
Figure 9 - Difference Spectrum Removing the Doublet-Doublet Triplet Interference of the Putative Luminol Derivative from the Second Silica Gel Pooled Fraction (see Fig. 6c, 6d and 6e)

It looks like a mixture or a complex substance.



PPM, Proton NMR

Figure 10 - Zone Electrophoresis of Immediate Diazotization Products



On further elution (fraction 14; Fig. 6g), still more new lines are observed. Thus, in agreement with capillary electrophoresis, there appear to be two major fragmentation products in the mixture, which appears to range from small molecules to a remnant of polymeric material.

Much better separations were obtained by means of TLC using silica gel plates, and it may be necessary to rely upon scaled-up preparative TLC in future studies so that compounds may be isolated with a degree of purity sufficient for structural studies. This approach would involve either solvent extractions of the fragmentation product or partial purifications on ordinary silica gel columns of the type described here, followed by preparative scale TLC. As an example, when the crude fragmentation product was extracted with pure dioxane and subjected to TLC using pure dioxane as the eluent, a yellow band migrating very near R_f 0.5 was observed. The TLC procedure was scaled up and about 6 mg of the substance was isolated and examined by ^1H -nmr, which revealed a single aromatic resonance at 9.1 ppm.

5. CONCLUSIONS AND RECOMMENDATIONS

Although the DALM polymer has been described as melanin-like, the chemical literature concerning melanin characterizations was not found to be helpful. Efforts to characterize the structures of the DALM and DAT polymers during previous funding periods, either by chemically fragmenting the fully developed polymer or by limiting the

degree of its polymerization, met with frustrations in spite of a considerable effort to find appropriate cleavage reagents (numerous candidate reagents were tried) or a means for truncating the polymer-forming reaction, but the situation was markedly improved during the current reporting period with the finding that aqua regia-like mixtures of HCl and HNO₃ effectively fragmented the polymer. It is expected that further progress in the structural characterization will depend upon obtaining low DP_n fragments of the DALM and DAT polymers, especially those in the dimer/trimer range since the polymeric backbone linkages should be revealed. Future studies should be focused almost entirely on a structural characterization of the DALM and DAT polymers since a better understanding of their luminescent and chemiluminescent properties will depend upon structural insight. A proportionately smaller effort might be devoted to a better characterization of DALM's sonochemiluminescence effects, but only if the structural characterizations proceed well.

Development of column chromatographic methods should involve true gradient elutions, a more uniform mesh size and taller columns in the 40 cm diameter range. The components eluting from low pressure liquid columns and observed by TLC and HPLC should be correlated. The department purchased a new Rainin HPLC instrument, but as noted, warranty problems (mostly computer associated) have delayed our access to it.

Preparative TLC separations have shown definite merit and will be used in conjunction with low pressure column

separations. The best approach identified at this point is to accomplish partial separations on low pressure silica gel columns, then further resolve the column fractions on preparative silica gel TLC plates. It is expected that HPLC will be brought into use in further studies of the fragmentation products.

In future work sufficiently purified samples of the resolved components of fragmented DALM will be submitted for elemental analysis and mass spectra. Such measurements were not attempted during the current period because nmr spectra revealed accompanying impurities. An elemental analysis of the insoluble barium form of the polymer will be obtained for an estimate of the mean equivalent weight of the anionic units.

The chemical processes of aqua regia fragmentation of DALM probably involve the species nitrosyl chloride (NOCl), which is known to form in the mixture of mineral acids (10). Future work should include a thorough literature survey of NOCl and its interactions with organic compounds.

The P.I. has known at length that solid state nmr techniques for line narrowing (i.e., by the cross-polarization/magic angle spinning method, CP/MAS, especially for observing carbon spectra) are applicable to direct characterizations of high molecular weight polymers, (13), including DALM, but the necessary equipment has not been available at this location. Very recently a state-of-the-art CP/MAS spectrometer was installed at Oklahoma State

University, and the continuing project will have access to it. We will also have the alternative option of using a 400 MHz CP/MAS instrument at Otsuka-Chemagnetics in Ft Collins, Colorado. However, it should be pointed out that the success of CP/MAS in characterizing DALM depends on the regularity or diversity of DALM's structure, which is still an unknown. If the structure is very diverse or random, the applicability of CP/MAS might be more limited.

6. REFERENCES AND FOOTNOTES

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(2) J. L. Kiel, G. J. O'Brien, J. Dillon and J. R. Wright, "Diazolumelanin: a Synthetic Luminescent Biopolymer", Free Radical Research Communications, 8, p115 (1990); The group at Brooks AFB has also noted product variability between seemingly identical preparations (private communications with Dr. John Kiel and Maj. Eric Holwit).

(3) Private communications with Dr. Johnathan Kiel at Brooks AFB, San Antonio, TX, and a Demonstration of the phenomenon on May 29, 1991. The aqueous solutions which exhibited the pulsed luminescence were described as 0.1 M with respect to Na_2CO_3 and contained H_2O_2 at 0.03% (w/v) and DADM at 0.05% (w/v).

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(10) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry", John Wiley Interscience, New York, NY, 1972, p179 and references.

(11) See Reference 1b, above, on page 22.

(12) J. R. Wright, "Chemiluminescent Probes Based on Luminol and Luminol Derivatives", Final Report for a USAF-UES Summer Faculty Research Program at Brooks AFB, Contract Number F49620-87-R-0004, August 3, 1988.

(13) J. R. Wright, W. A. Hendrickson, S. Osaki and G. T. James, "Physical Methods for Inorganic Biochemistry", "Biochemistry of the Elements Series", Vol. 5, E. Frieden, Editor, Plenum Press, New York, 1986, Chapter 2.

7. APPENDIX I - DEVELOPMENT OF STUDENTS

The guide literature does not require this section, but it was included since research findings were not the only direct product of the project. In particular, the project's contribution to human resources were significant and should be taken into account.

The technician, Doreatha Powell, and two of the students involved in the project, Ken Whittington and Gene McDaniel, aspire to pursue a medical degree. Ken is now in his second semester of medical school and doing well. Gene has been accepted and will start in August of 1995. Doreatha is now on a final acceptance list and apparently will also enter a medical program next summer. The most recent student, Kris Wood, is one of our best academic performers and may choose a Ph.D. chemistry career. He will continue if the project resumes in April of 1995. All of these individuals are grateful for the opportunity to participate in a basic research project.

APPENDIX II - PUBLICATIONS

There have been no publications since the last report (reference 1b, above). The P.I. is working on a paper concerning DALM sonochemiluminescence, but it would not be prudent to consider a submission until some reproducibility problems are cleared up. Sonochemiluminescence was observed with the polymer in the presence of formate ion, but not all of the formate preparations produced the effect - apparently the result of an impurity in some formate sources. It is too early to consider a paper concerning the structure of DALM.